In vitro endosome-lysosome transfer of dephosphorylated EGF receptor and Shc in rat liver

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Abstract **We have studied the endosome-lysosome transfer of internalized epidermal growth factor receptor (EGFR) complexes in a cell-free system from rat liver. Analytical subfractionation of a postmitochondrial supernatant fraction showed that a pulse of internalized [^{125}I]EGF was largely associated with a light endosomal fraction devoid of lysosomal markers. After an additional 30 min incubation in vitro in the presence of an ATP-regenerating system, the amount of [^{125}I]EGF in this compartment decreased by 39%, with an increase in [^{125}I]EGF in lysosomes. No transfer of [^{125}I]EGF to the cytosol was detected. To assess the fate of the internalized EGFR protein over the time course of the endo-lysosomal transfer of the ligand, the effect of a saturating dose of native EGF on subsequent lysosomal targeting of the EGFR was evaluated by immunoblotting. A massive translocation of the EGFR to the endosomal compartment was observed in response to ligand injection coincident with its tyrosine phosphorylation and receptor recruitment of the tyrosine-phosphorylated adaptor protein Shc. During cell-free endosome-lysosome fusion, a time-dependent increase in the content of the EGFR and the two 55- and 46-kDa Shc isoforms was observed in lysosomal fractions with a time course superimposable with the lysosomal transfer of the ligand; no transfer of the 66-kDa Shc isoform was detected. The relationship between EGFR tyrosine kinase activity and EGFR sorting in endosomes investigated by immunoblot studies with anti-phosphotyrosine antibodies revealed that endosomal dephosphorylation of EGFR and Shc preceded lysosomal transfer. These results support the view that a lysosomal targeting machinery distinct from the endosomal receptor kinase activity, such as the recruitment of the signaling molecule Shc, may regulate this sorting event in the endosome.**

Key words: Endocytosis; Organelle fusion; Intracellular signaling; Tyrosine phosphorylation

1. Introduction

In mammals, the highest concentration of the epidermal growth factor receptor (EGFR) is found in liver parenchyma [1]. The hepatic EGFR is believed to be involved in liver regeneration via mitogenic signaling pathways [2]. Ligand mediated activation of the EGFR leads to a number of subcellular events including the recruitment and tyrosine phosphorylation of Shc and the recruitment of Grb2 followed by the activation of p21^{ras} and downstream pathways [3]. Following ligand binding and the initiation of signal transduction pathways, the EGFR is rapidly trafficked to coated pits, internalized and ultimately degraded when endosomes fuse with lysosomes [4,5].

While receptor tyrosine kinases (RTKs) such as those for EGF and PDGF are targeted largely to lysosomes for degradation others such as the RTKs for insulin and IGF-I are recycled largely to the plasma membrane [6]. Hence, a machinery must exist in the endosome to sort different receptors into distinct vesicle populations. Data on the compartmentalization of the signaling responses of the EGFR in liver parenchyma support the view that the RTK activity is important for ligand-induced sorting of this receptor to the pathway for lysosomal degradation [7,8]. Thus, the EGFR is maximally tyrosine-phosphorylated following internalization into endosomes as a consequence of a partial dissociation and degradation of internalized EGF within endocytic vesicles [4,5,9]. We have recently demonstrated that the cysteine protease cathepsin B down-regulates internalized EGF-EGFR complexes within endosomes by inducing proteolytic cleavages that yield C-terminal truncated forms of EGF [5]. EGFR recruitment and tyrosine phosphorylation of endosomal substrates may also regulate these sorting events. The complex of tyrosine-phosphorylated Shc and Grb2 represents a relevant candidate at this locus [7,10].

We have recently developed a method for assaying endosome-lysosome transfer of internalized ligand-receptor complexes in hepatic tissue [11]. We have shown that in a liver cell-free system containing in vivo endocytosed insulin, glucagon and GalBSA, these ligands are transferred in vitro from endosomes to lysosomes [11]. A low lysosomal transfer of the α and β subunits of the insulin receptor also occurred during endosome-lysosome interaction [11]. In the present work, we used the in situ liver model system for endosome-lysosome transfer to study (a) the endosomal fate of the internalized EGF-EGFR complex; (b) the phosphorylation state of the internalized EGFR during the fusion process; and (c) the fate of EGFR-associated Shc, a major physiological substrate for the EGFR tyrosine kinase.

2. Materials and methods

2.1. Peptides, ligand radioiodination, antibodies, protein determination, enzyme assays and materials

Mouse EGF (receptor grade) was purchased from Collaborative Biomedical. [^{125}I]EGF (400–500 Ci/mmol) was prepared using chloramine T and purified by gel filtration on Sephadex G-50. Monoclonal horseradish peroxidase (HRP)-conjugated antibody raised to phos-

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Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; Shc, src homology 2 (SH2) domain-containing α2 collagen-related; RTK, receptor tyrosine kinase; TCA, trichloroacetic acid

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photyrosine was purchased from Amersham. Polyclonal antibody raised to the SH2 domain (residues 366–473) of Shc and polyclonal antibody to a synthetic peptide (residues 1164–1176) of the EGFR used for immunoblotting were obtained from Dr. J.J.M. Bergeron (McGill University, Montréal, Que., Canada) [7]. Rabbit anti-rat procathepsin B and rabbit anti-mouse cathepsin D were obtained from Dr. John S. Mort (Shriners Hospital for Crippled Children, Montréal, Que., Canada) [12]. HRP-conjugated goat anti-rabbit IgG was from Bio-Rad. The protein content of isolated fractions was determined by the method of Lowry et al. [13]. Galactosyltransferase was assayed as described by Beaufay et al. [14]. Acid phosphatase was assayed as described by Trouet [15]. 5′-Nucleotidase was assayed as described by Heppel and Hillmoe [16]. Enhanced chemiluminescence (ECL) detection kit was from Amersham. Polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad. All other chemicals were obtained from commercial sources and were of reagent grade.

2.2. Animals and injections

Male Sprague-Dawley rats, body weight 180–200 g, were obtained from Charles River France (St. Aubin Les Elbeufs, France) and were fasted for 18 h prior to killing. Native EGF (5 μg/100 g body weight) or [^{125}I]EGF (20×10^6 cpm) were diluted in 0.3 ml of 0.15 M NaCl and injected within 5 s into the penis vein under light ether anaesthesia.

2.3. Isolation of subcellular fractions from rat liver and transfer reaction

Subcellular fractionation was performed using established procedures [17–19]. Animals were killed 10 min after injection of the appropriate ligand and livers rapidly removed and minced in either isotonic ice-cold homogenization buffer containing 0.25 M sucrose, 10 mM TES pH 7.4 and 7 mM $MgCl_2$. Intact cells, nuclei and mitochondria were removed from the homogenate by centrifugation at $33\,000\times g$ for 1 min. The cell-free transfer reaction was carried out as described previously by Chauvet et al. [11]. The postmitochondrial supernatant (referred to as LPS fraction) was incubated at 4°C or 37°C, and, where indicated, in the presence of 5 mM ATP, 1 mg/ml creatine kinase and 20 mM phosphocreatine. After cooling to 4°C, incubation mixtures were layered onto 10 ml linear gradients prepared with 35% (mass/vol.) Nycodenz and 0.25 M sucrose containing 10 mM TES pH 7.4 and 2 mM EDTA (density range, 1.06–1.16 g/ml). Gradients were centrifuged at $200\,000\times g$ for 60 min in a Beckman SW 41 rotor, following which 0.5 ml fractions were collected, densities determined and enzyme activity, radioactivity and immunoblot analyses carried out. In some experiments the integrity of radiolabelled EGF was assessed by precipitation with trichloroacetic acid (TCA) [11,12,17–19].

2.4. Immunoblot studies

Electrophoresed samples were transferred to nitrocellulose blots (0.45 μm) for 60 min at 380 mÅ in transfer buffer containing 25 mM Tris-base and 192 mM glycine. The blots were incubated for 3 h with 5% skim milk or 2% bovine serum albumin (for phosphotyrosine immunoblots) in 10 mM Tris-HCl pH 7.5, 300 mM NaCl and 0.05% Tween-20. The blots were then incubated with primary antibody (affinity-purified polyclonal antibodies to Shc diluted 1:5000, EGFR diluted 1:5000, monoclonal anti-phosphotyrosine diluted 1:2500, polyclonal anti-procathepsin B diluted 1:200 and polyclonal anti-cathepsin D diluted 1:200) in the above buffer for 16 h at 4°C. After incubation, the blots were washed three times with 0.5% skim milk or 0.2% bovine serum albumin (for phosphotyrosine immunoblots) in 10 mM Tris-HCl pH 7.5, 300 mM NaCl and 0.05% Tween-20 over a period of 1 h at room temperature. The bound immunoglobulin was detected using HRP-conjugated goat anti-rabbit IgG. Bands were detected using ECL and quantified by densitometric scanning of the X-ray film of corresponding immunoblots.

3. Results

We have previously reported the steady-state distribution of cathepsins in liver parenchyma detected with sensitive and specific antibodies [12]. Immunoblotting procedures performed on highly enriched hepatic subfractions have indicated that procathepsin B is membrane-bound in hepatic endosomes whereas mature cathepsin D is mainly recovered in lysosomes. We therefore used the well-characterized anti-cathepsin antibodies to assess the distribution of endosomes and lysosomes throughout the hepatic LPS fraction which was subfractionated on a linear Nycodenz gradient (Fig. 1C). Moreover, other classical marker enzymes have been also assessed (Fig. 1A and B). Immediate analysis of LPS fraction on Nycodenz gradients showed that the Golgi marker galactosyltransferase and the endosomal procathepsin B enzyme were found in low density fractions between 1.075–1.105 g/ml whereas the lysosomal markers acid phosphatase and cathepsin D were found in high density fractions between 1.11–1.14 g/ml (Fig. 1A and C). 5′-Nucleotidase, a plasma membrane marker, migrated as a slightly denser peak (peak density $\approx$ 1.105 g/ml) than the Golgi marker galactosyltransferase (Fig. 1A). The distribution of marker enzymes was almost unchanged when the LPS fraction was incubated at 37°C for 30 min in the presence of ATP and an ATP-regenerating system (Fig. 1B). Thus, components appearing at densities 1.075–1.105 and 1.11–1.14 g/ml were scored, respectively, as truly endosomal and lysosomal.

Next, we examined the subcellular distribution of internalized [^{125}I]EGF associated with the LPS fraction over time (Fig. 2A). Rats were administered an intravenous injection of [^{125}I]EGF and killed 10 min after injection, when most of the ligand would be located in the endosomes [4,5,20]. Analytical subfractionation on Nycodenz gradients of the postmitochondrial supernatants prepared from these livers showed that the radioactivity appeared in a single broad peak of density $\approx$ 1.075–1.105 g/ml (Fig. 2A) clearly distinguished from the peak of activity of the lysosomal enzyme markers acid phosphatase and cathepsin D (see Fig. 1). Analysis of the LPS fraction after a 30 min incubation at 37°C in the presence of ATP and an ATP-regenerating system showed that 24% of the radioactivity had moved to a peak density 1.11–1.14 coinciding with the peak for the lysosomal enzyme markers (see Fig. 1). Omission of ATP and an ATP-regenerating system prevented any increase in radiolabel in the lysosomal position after 30 min at 37°C (Fig. 2A).

To evaluate the contribution of endosomal and lysosomal components to [^{125}I]EGF degradation, the integrity of the ligand was assessed by precipitation with TCA (Fig. 2B). With the LPS fraction kept at 4°C, more radioactivity was present in the TCA-soluble form in high density lysosomal components ($\approx$40%) than in low density endocytic structures ($\approx$33%). Incubation at 37°C produced a rapid increase in acid-soluble radioactivity recovered at the top of the gradient suggesting that only small size breakdown products diffused out of the membrane endo-lysosomal compartment.

The time course of transfer of the EGFR from the endosomal to the lysosomal position was next examined by density gradient analysis of the LPS fractions prepared from control and EGF-injected rats (Fig. 2C). With control LPS fractions prepared from non-injected rats, most of the EGFR was found in the middle of the gradient between densities 1.080–1.115 g/ml which coincided with 5′-nucleotidase, the plasma membrane marker (see Fig. 1A and B). With LPS fractions prepared from native EGF-treated rats and incubated at 4°C, most of the EGFR appeared in the endosomal region of the gradient (1.075–1.100 g/ml). When the LPS fraction was incubated at 37°C for 15 min, the EGFR found in the endosomal region of the Nycodenz gradient was markedly reduced as would be expected if some EGFR was being degraded

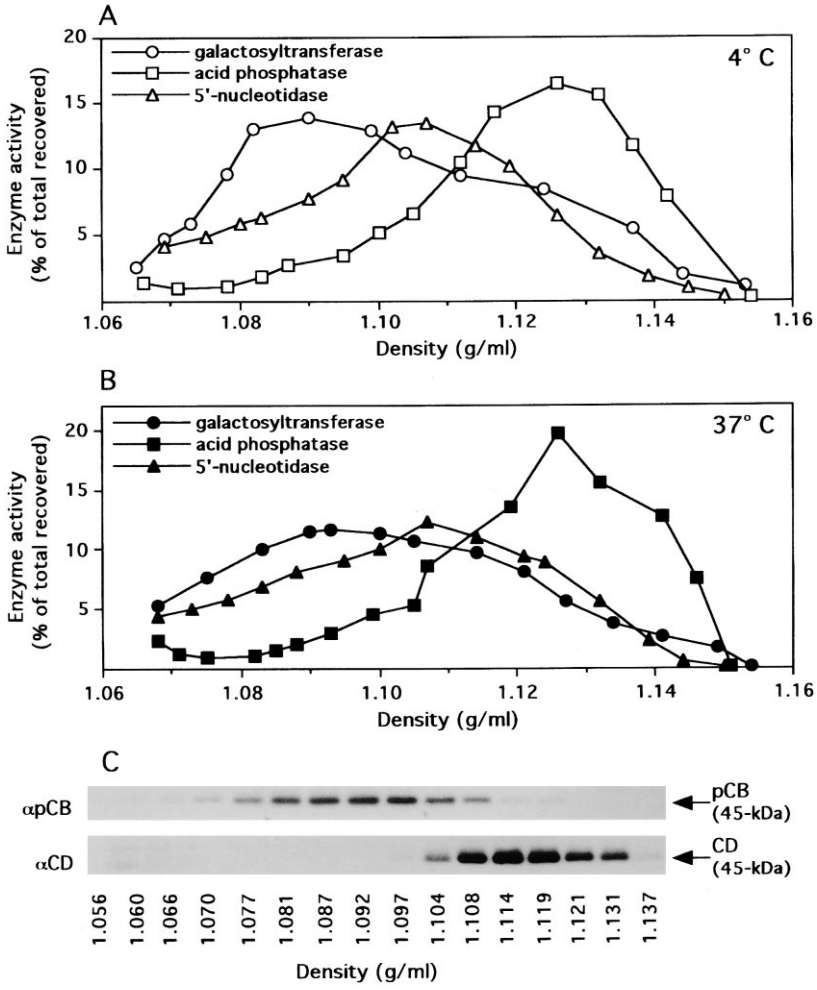

Fig. 1. Density distribution of marker enzymes associated with the LPS fraction on Nycodenz gradients. The LPS fraction was isolated from control rats and immediately subfractionated on linear Nycodenz density gradients (panels A and C), or incubated with ATP and an ATP-regenerating system at 37°C for 30 min (panel B). Panels A and B, galactosyltransferase (circles), acid phosphatase (squares) and 5′-nucleotidase (triangles) activities were determined and results expressed as a percentage of total enzymatic activity recovered. Panel C, the content of procathepsin B (pCB) and cathepsin D (CD) was evaluated by immunoblotting with polyclonal antibodies. Thirty μl of each subfraction were loaded onto each lane. Bands were visualized by immunostaining with HRP-conjugated secondary antibody.

enzymatically at this subcellular site [5,21]. After a 30 min incubation at 37°C, the EGFR distributed into two peaks. The majority of the EGFR (81%) appeared in the endosomal peak whereas a small fraction appeared in a dense peak (1.127–1.132 g/ml) consistent with lysosomal compartmentation. No detectable amounts of EGFR movement occurred in the absence of ATP and an ATP-regenerating system.

The EGFR phosphorylation state was next assessed by immunoblotting with monoclonal anti-phosphotyrosine antibodies (Fig. 3). A strong phosphotyrosine content of the endosomal EGFR was observed when the LPS fraction was incubated at 4°C, as well as the presence of the tyrosine-phosphorylated 55-kDa form of Shc (Fig. 3A). Fig. 3B depicts the decline in phosphotyrosine content of the EGFR and 55-kDa Shc isoform after a 10 min incubation of the LPS fraction at 37°C. In each case, there was a major loss of phosphotyrosine content, with an immunoreactivity barely perceptible with the endosomal EGFR. We conclude that a rapid EGFR phosphotyrosine dephosphorylation reaction was occurring in endosomes before the endo-lysosomal transfer event.

To evaluate the relationship between compartmentalization and signaling, we isolated Nycodenz subfractions and evaluated them for the presence of the EGF-associated signaling molecule Shc as a consequence of ligand administration (Fig. 4). With control LPS fractions prepared from non-injected rats and subjected to this procedure without warming (4°C/ATP panel), only a small amount of the 55-kDa Shc isoform migrated within the density range for endosomal markers (density 1.075–1.105 g/ml), the vast majority of the three Shc isoforms being recovered at a position corresponding to the soluble cytosolic pool (density < 1.070 g/ml). The density distribution of Shc was unchanged if control LPS fractions were incubated at 37°C for 30 min with an energy source (30 min 37°C/ATP panel). In response to EGF stimulation (EGF 4°C/ATP panel), a partial recruitment of the 55-kDa protein Shc and also, but to a lesser degree, the 44-kDa protein Shc, was mainly found in the light endosomal vesicles (density 1.075–1.105 g/ml). In contrast, when the LPS fractions prepared from EGF-injected rats were warmed to 37°C for 15 min (EGF 15 min 37°C/ATP panel) or 30 min (EGF 30 min 37°C/ATP panel), a marked increase in both the 55- and 44-kDa Shc isoforms was detected in the light endosomal

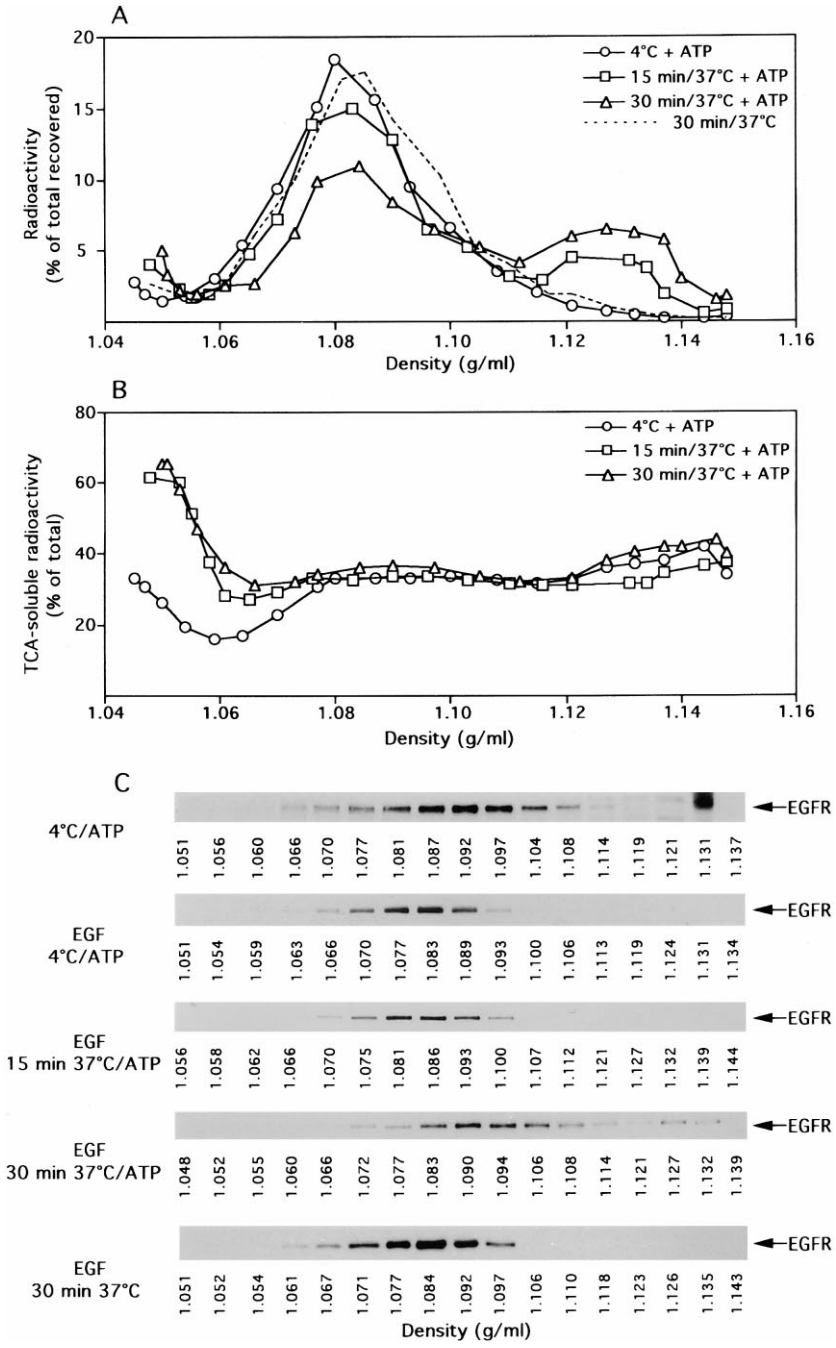

Fig. 2. Time course of transfer of [^{125}I]EGF and EGFR from the endosomal to the lysosomal position on Nycodenz gradients. LPS fractions were isolated 10 min after [^{125}I]EGF administration (10^7 cpm; panels A and B) or native EGF administration (5 μg/100 g body weight; panel C). Panels A and B, LPS fractions were incubated with ATP and an ATP-regenerating system at 4°C for 30 min (circles), or 37°C for 15 min (squares), or 37°C for 30 min (triangles), and in the absence of ATP at 37°C for 30 min (dotted line; panel A). In panel A, results are expressed as percentages of the total radiolabel on the gradient. In panel B, the amount of degraded [^{125}I]EGF was determined by precipitation with trichloroacetic acid. In panel C, the EGFR content was evaluated in each subfraction by immunoblotting with polyclonal antibody. The arrows on the right indicate the mobility of the EGFR (≈170 kDa). Twenty μl of each subfraction were loaded onto each lane. Bands were visualized by immunostaining with HRP-conjugated secondary antibody.

(15 min) and dense lysosomal (30 min) fractions (densities 1.075–1.105 g/ml and 1.11–1.14 g/ml, respectively). We were unable to detect any association of the 66-kDa Shc protein with either low (endosomal) or high (lysosomal) density fractions. The endo-lysosomal transfer of the 55- and 44-kDa Shc isoforms was not observed with the cell-free system incubated without ATP and an ATP-regenerating system (EGF 30 min 37°C panel).

4. Discussion

These biochemical studies have enabled the testing of three hypotheses concerning EGFR sorting in hepatic endosomes: (a) the EGFR undergoes endosome-lysosome transfer in vitro along with EGF with comparable kinetics; (b) the internalized EGFR accesses lysosomes under a dephosphorylated state; and (c) a co-transfer of the 55- and 44-kDa Shc isoforms

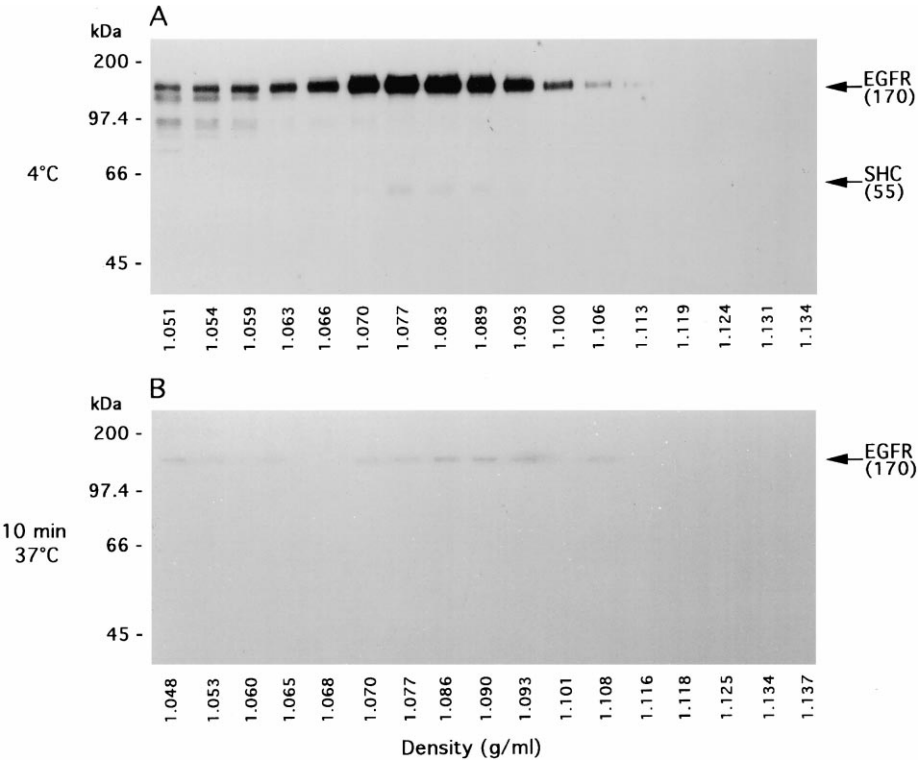

Fig. 3. Tyrosine-phosphorylation state of EGFR and Shc during the in vitro endosome-lysosome transfer. LPS fractions were isolated 10 min after EGF administration (5 μg/100 g body weight), incubated with ATP and a regenerating system for 10 min at 4°C (panel A) or 37°C (panel B) and subfractionated on linear Nycodenz density gradients. Each subfraction (150 μl) was evaluated by Western-blotting for their immunoreactivity with HRP-conjugated monoclonal antibody against phosphotyrosine. The arrows on the right indicate the mobility of the EGFR (≈170 kDa) and the 55-kDa isoform of Shc. Control experiments have shown that the polyclonal anti-Shc antibody immunoprecipitated the 55-kDa endosomal tyrosine-phosphorylated protein detected in the anti-phosphotyrosine immunoblot (results not shown).

occurs during the fusion process. To the best of our knowledge, this is the first such a test of these hypotheses with an in vitro fusion system.

Using the in situ liver model system for endosome-lysosome fusion, we clearly show a transfer of EGFR through and an association of Shc with the endo-lysosomal pathway in response to EGF. The endo-lysosomal transfer of the internalized EGFR and the adaptor protein Shc was (i) ligand-dependent (it was only observed with the LPS fractions prepared from EGF-treated rats); (ii) temperature-dependent (it required a 37°C incubation); and (iii) energy-dependent (it required ATP and an ATP-regenerating system). Moreover, it was not due to non-specific aggregation of organelles during incubation of LPS supernatants since no major change in the density distribution of marker enzymes was observed during the fusion process. Finally, the time course of the process is consistent with the time taken for EGF to appear in lysosomes in whole liver [4] and in Hep-2 cells [22]. The similar kinetics of lysosomal appearance of the internalized EGFR and the EGFR-associated signaling molecule Shc suggested an extended signal transduction consequent to ligand-mediated EGFR translocation to the lysosomal apparatus [8].

Using cell-free experiments originally developed for asialofetuin, a ligand of the asialoglycoprotein receptor [23], an in vitro model for the transfer of endocytosed EGF-EGFR complexes to lysosomes is proposed. As with asialofetuin, GalBSA, glucagon and insulin [11,23,24], the cell-free transfer of EGF-EGFR complexes occurred from endosomes isolated at a late stage of endocytosis (time of killing 10 min; [7,20]) and required the addition of cytosol and an ATP-regenerating system [11,23]. The endosome-lysosome transfer was accompanied by increased low molecular weight degradation products of EGF at the upper part of the gradient. With respect to internalized EGFR, no immunoreactive intermediate breakdown products were detected in our study within both endosome and lysosome compartments (results not shown). These data suggest that, as previously reported using the in situ liver model [5,21], internalized EGFR is rapidly degraded by a proteolytic process that destroys the epitopes recognized by anti-EGFR antibodies.

Although an endosome-lysosome transfer of these ligands was clearly established with the liver cell-free system, the endosomal sorting of these polypeptides differs in several respects: (a) EGF (this study) and asialofetuin [23] transfer was maximal at 30 min, as compared to 10 min for insulin and glucagon [11]; (b) lysosomal recovery after a 10 min incubation at 37°C was low in the presence of insulin ($<12\%$), moderate with EGF and glucagon (15–20%) and high with GalBSA and asialofetuin (20–25%) [11,23]; and (c) the relative amount of receptor recovered at the lysosomal position during endosome-lysosome interaction was high with EGF (this study) and very low with insulin [11].

Membrane proteins destined for the lysosome display some form of trafficking signal [25]. Sequences containing proposed lysosomal targeting information and independent of the tyrosine kinase have been identified in the cytoplasmic domain of the EGFR between residues 945–958 and 1022–1063 [26,27]. Other studies suggested that the tyrosine kinase itself plays a

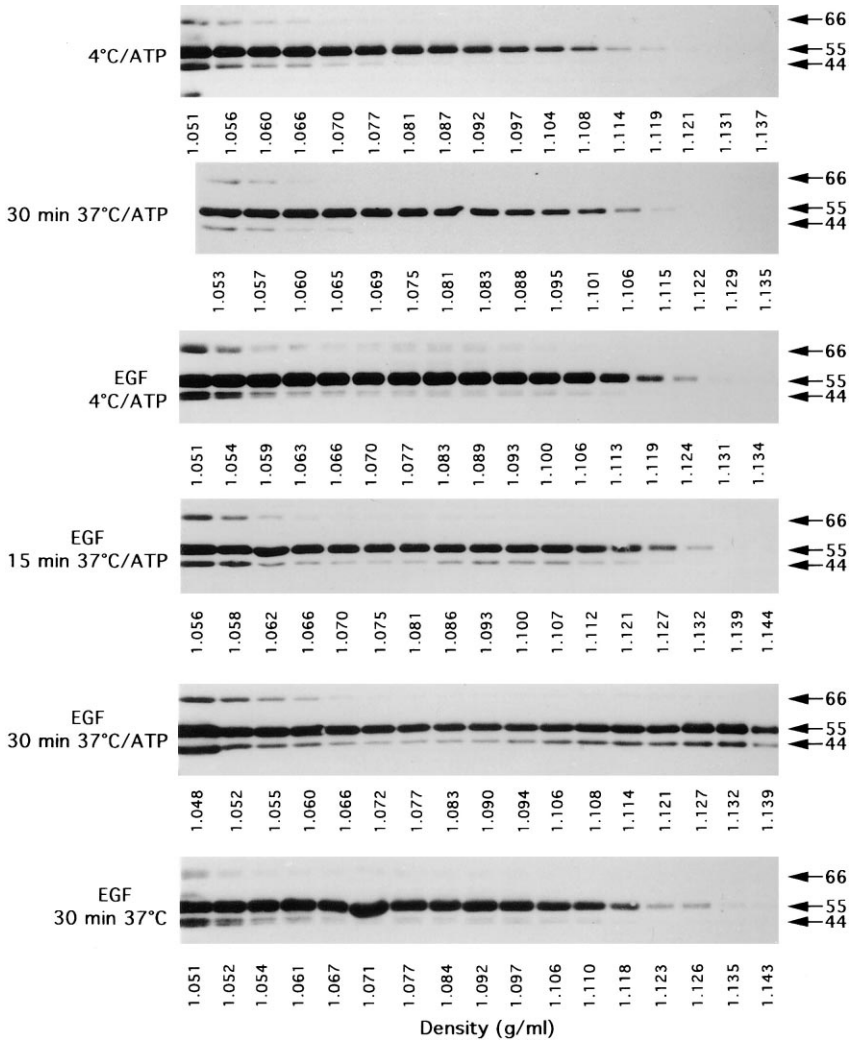

Fig. 4. Time course of endosome-lysosome transfer of Shc isoforms after EGF administration. LPS fractions were isolated from control rats (4°C/ATP and 30 min 37°C/ATP panels) or from rats having received an EGF injection (5 μg/100 g body weight; EGF panels). LPS fractions were immediately subfractionated on linear Nycodenz density gradients (4°C/ATP panel) or were incubated without ATP at 37°C for 30 min (EGF 30 min 37°C panel) or with ATP and an ATP-regenerating system at 4°C for 30 min (EGF 4°C/ATP panel), or 37°C for 15 min (EGF 15 min 37°C/ATP panel), or 37°C for 30 min (EGF 30 min 37°C/ATP panel). The content of Shc isoforms was evaluated in each subfraction by immunoblotting with polyclonal antibody. Fifty μl of each subfraction were loaded onto each lane. Bands were visualized by immunostaining with HRP-conjugated secondary antibody.

role in generating a lysosome targeting signal [27,28]. In accordance with this hypothesis, a greater and prolonged EGFR tyrosine phosphorylation has been demonstrated in rat liver endosomal membranes compared to the EGFR located at the plasma membrane [5,7,29,30]. The high phosphorylation content of internalized EGFR has been ascribed to a low dissociation and degradation state of EGF within the endosome [4,5,31]. However, shortly after, endosomal dephosphorylation induced by closely associated phosphotyrosine phosphatase(s) then leads to the deactivation of the EGFR's exogenous kinase activity [32]. Our results showing that the half-time for EGFR dephosphorylation within endosomes (<5 min) was shorter than that for lysosomal transfer of endosomal EGF-EGFR complexes (>15 min) suggest that the receptor tyrosine kinase activity appeared to have only a small effect (if any) on the sorting pattern with our in vitro endo-lysosome fusion system. However, the rapid loss of EGFR phosphotyrosine content occurring in endosomes observed in the present study is compatible with a previous observation of EGFR dephosphorylation observed using cell-free endosomes [32] and the in situ liver model [5,7]. Moreover, the data that receptor mutants lacking an ATP-binding site or an entire kinase domain are degraded as fast as wild-type receptors have also questioned the direct involvement of tyrosine kinase activity in the endosomal sorting of EGFRs [26,33]. Finally, another study on EGF recycling at different levels of receptor occupancy showed that the regulatory role of receptor tyrosine kinase can be revealed at low EGF concentrations when the lysosome targeting apparatus is not saturated [33]. Thus, it is also conceivable that following administration of a saturating dose of EGF (5 μg/100 g body weight; [20,29]) some function of receptor kinase activity may be reduced.

Tyrosine phosphorylation of endosomal proteins and/or the recruitment of proteins to the endosome may also mediate lysosomal targeting [25]. Endosomal RTK tyrosine phosphorylation of annexin-1 has been proposed as the mechanism by which the EGFR at the periphery of the endosomal mem-

brane is sorted into the inner vesicles of multivesicular endosomes and then subsequently targeted for degradation in lysosomes [34]. Tyrosine-phosphorylated Shc and Grb2 also represent relevant candidates at this locus [7,10,35]. The sequential endosomal and lysosomal association of the 44- and 55-kDa Shc isoforms in response to EGF suggests that Shc could be the missing molecular link between endocytic and lysosomal vesicles. Upon EGF treatment and RTK activation, Shc proteins, as analyzed by preparative subcellular fractionation [7] or by immunofluorescence and immunoelectron microscopy [35], were found to be associated with the cytosolic surface of endocytic structures, such as coated pits and endosomes, and with the peripheral cytosol. In the present study, we confirmed such an intracellular localization for the adaptor protein, and also, we provided the first evidence for a lysosomal localization of the 44- and 55-kDa Shc isoforms in response to EGF. Consistent with our data, Okada et al. [36] have shown that the predominant EGF-dependent Shc signaling pathway occurs via both the 55- and 44-kDa Shc species. Thus, the massive recruitment of Shc proteins to lysosomal structures suggests a new function for the adaptor protein in RTK signal transduction and membrane trafficking.

The influence of endosomal proteolysis on the steady-state distribution of cognate receptor has been previously inferred from a comparison of insulin, TGFα and EGF receptor internalization [8,9,37]. Insulin [19,38] and TGFα [37] are subject to rapid endosomal dissociation from their receptors and subsequent degradation whereas EGF is selectively resistant to dissociation and is only partially degraded in the endosome [4,5]. The differences in endosomal dissociation and degradation of these ligands after internalization coincided with altered receptor trafficking and receptor phosphotyrosine profiles through the endosomal/lysosomal pathway [5,7,37,38]. Thus, although EGF, TGFα and insulin have similar internalization kinetics, the targeting of the receptor for degradation in lysosomes (down-regulation) was achieved more effectively by EGF [11,29,37]. However, an EGF receptor-like response in tyrosine phosphorylation of the internalized insulin receptor was observed if the ligand used was the insulin analogue H2, a genetically engineered analogue which displays a reduced rate of proteolysis in endosomes as compared to authentic insulin [38]. Moreover, Hansen et al. [39] observed Shc phosphorylation in response to insulin when administered to CHO cells overexpressing the insulin receptor, and this effect was augmented in response to the analogue H2. Studies are underway to determine whether, in response to the insulin analogue H2, the subsequent extension of the temporal window of insulin receptor activation in the endosome [38], as well as enhanced Shc tyrosine phosphorylation [39], cause a translocation of both insulin receptor and Shc to the lysosomal compartment.

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